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Evaluation of Disk Diffusion Method for Determining Posaconazole Susceptibility of Filamentous Fungi: Comparison with CLSI Broth Microdilution Method

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The disk diffusion method was evaluated for determining posaconazole susceptibility against 78 strains of molds using two culture media in comparison with the CLSI (Clinical Laboratory Standards Institute) broth microdilution method (M38-A). A significant correlation between disk diffusion and microdilution methods was observed with both culture media.

Invasive infections due to *Aspergillus* spp. and other filamentous fungi (molds) have emerged as prominent causes of morbidity and mortality worldwide in immunocompromised hosts (2, 6–8, 14). Posaconazole is a water-insoluble investigational triazole with in vitro and in vivo activity against yeasts and molds (1, 3, 11, 15). The Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards) has developed a reference method for microdilution antifungal susceptibility testing of filamentous fungi (CLSI/NCCLS M38-A) (9). Agar-based methods such as Etest and disk diffusion can be good alternatives because they are simpler and faster than broth-based methods. Some investigators have explored the use of Etest susceptibility testing for posaconazole against filamentous fungi (4, 12), but data on posaconazole testing against molds by the disk diffusion method are lacking. In this study, we investigated the applicability of a disk diffusion method for testing the susceptibility of posaconazole against molds in order to compare the results that were obtained with this assay with those obtained by the broth microdilution method and to evaluate the influence of culture media.

A total of 78 clinical isolates were tested: 57 *Aspergillus* spp. (20 *Aspergillus terreus*, 16 *Aspergillus fumigatus*, 14 *Aspergillus flavus*, 4 *Aspergillus niger*, and 3 *Aspergillus glaucus)* and 21 other filamentous fungi (7 *Rhizopus* spp., 3 *Mucor* spp., 5 *Scedosporium apiospermum*, 3 *Scedosporium prolificans*, and 3 *Fusarium* spp.). These isolates were recovered from clinical specimens received in Valme University Hospital in Seville (Spain) and the La Fé University Hospital in Valence (Spain). The identification of each strain was performed using conventional mycological techniques. The mold isolates were maintained in sterile water and were subcultured on antimicrobial-agent-free potato dextrose agar (Difco) to ensure viability and purity. Stock inoculum suspensions were prepared from 7-day-old cultures grown on potato dextrose agar by following CLSI guidelines (document M38-A) (9). Stock suspensions were adjusted spectrophotometrically to optical densities that ranged from 0.09 to 0.11 (80 to 82% transmittance) and contained conidia or sporangiospores and hyphal fragments. The diluted (2×) inoculum sizes ranged from 0.9×10^4 to 4.7×10^4 CFU/ml, as demonstrated by a quantitative colony count on Sabouraud dextrose agar. The same inoculum was used for broth and agar methods.

The broth microdilution test was done in accordance with CLSI guidelines for conidium filamentous fungi (CLSI document M38-A) (9). Posaconazole was obtained from the Schering-Plough Research Institute (Kenilworth, N.J.), and it was dissolved in 100% dimethyl sulfoxide. The MICs were determined after 48 h of incubation. The MIC endpoints were evaluated for the lowest drug concentration that showed a prominent reduction of the growth control in the control well (≥50% or MIC-2) and the lowest drug concentration that showed absence of growth or complete growth inhibition (100% inhibition or MIC-0) (5).

The procedure for the disk diffusion method was mainly that of CLSI document M44-A (10). Posaconazole disks (5 μg, Oxoid) were used. The following culture media were tested: RPMI agar with 2% glucose (RPMI-G; Izasa, Spain) and Mueller-Hinton agar (Difco) supplemented with 2% glucose and methylene blue (0.5 mg/ml) (MHB) (10). The plates were incubated at 35°C and read at 24 and 48 h. The inhibition zones (IZs, in millimeters) were read at the point of marked decrease in fungal density (IZ-2) and the point of complete inhibition of growth (IZ-0).

A. fumigatus ATCC 204305, *A. flavus* ATCC 204304, *Candida parapsilosis* 22019, and *Candida krusei* 6258 were included in each susceptibility test for quality control and assessment of reproducibility.

For analysis of the results, geometric means (GM) and ranges of MICs and arithmetic means and ranges of IZ were calculated. To determine the correlation between the disk diffusion method and the microdilution method, the IZs were plotted against their respective MICs. Pearson's correlation coefficient was used to calculate a regression line for each comparison.

Table 1 shows the results of microdilution at 48 h and disk

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TABLE 1. In vitro activity of posaconazole against 78 strains of filamentous fungi[a]

Species (no. of strains)	Medium	Disk diffusion, IZ-0 (mm)		Microdilution, MIC-0 (μg/ml)	
		Range	AM[b]	Range	GM
Rhizopus spp. (7)	RPMI-G	18–22	19.7	0.06–2	0.31
	MHB	10–20	14.8		
Mucor spp. (3)	RPMI-G	12–18	15.3	0.25–0.5	0.31
	MHB	11–23	17.3		
A. terreus (20)	RPMI-G	30–37	31.7	0.015–0.25	0.073
	MHB	29–31	30		
A. fumigatus (16)	RPMI-G	22–35	29.1	0.03–0.25	0.079
	MHB	20–32	27.1		
A. flavus (14)	RPMI-G	15–32	27.4	0.03–0.25	0.081
	MHB	20–35	27		
A. niger (4)	RPMI-G	26–32	27.7	0.06–0.125	0.086
	MHB	23–26	24.7		
A. glaucus (3)	RPMI-G	27–30	27.6	0.06–0.25	0.06
	MHB	26–27	25.6		
Fusarium spp. (3)	RPMI-G	0	0	8	8
	MHB	0	0		
S. prolificans (3)	RPMI-G[c]	0	0	>8	>8
	MHB	0	0		
S. apiospermum (5)	RPMI-G[c]	0–35	22	0.5–1	1
	MHB	0–17	27		

[a] Activity was determined by the disk diffusion method testing two media (24 h of reading) and the microdilution method (48 h of reading).
[b] AM, arithmetic media of the inhibition zone diameters.
[c] Results reflect a reading at 48 h.

diffusion at 24 h except for *Scedosporium* spp., whose disk diffusion test results could be not interpreted at 24 h because of poor growth. Disk diffusion test results for *Mucorales* could not be interpreted in RPMI agar and MHB at 48 h because they showed overgrowth, and no inhibition zone was observed. For *Aspergillus* spp., *Fusarium* spp., and *S. prolificans*, the MIC-0 and the IZ-0 were in agreement with the MIC-2 and IZ-2, respectively, while for *Mucorales*, a difference of 2 to 10 mm between the IZ-0 and IZ-2 was observed. The GM of the MICs were low for *Aspergillus* spp. and *Mucorales* (≤0.31 μg/ml) and for *S. apiospermum* (≤1); for *Fusarium* spp. and *S. prolificans*, the GM of the MICs were high (≥8 μg/ml). The MIC results for control strains that were obtained by the microdilution method at 48 h were 0.03 μg/ml for *C. parapsilosis* 22019, 0.25 μg/ml for *C. krusei* 6258, 0.06 μg/ml for *A. fumigatus* ATCC 204305, and 0.06 μg/ml for *A. flavus* ATCC 204304. These results are within the MIC range limits recommended by the M38-A document (9). The respective MIC results that were obtained by the disk diffusion method at 24 h in two media, RPMI-G and MHB, were 34 and 30 mm for *C. parapsilosis* 22019, 32 and 27 mm for *C. krusei* 6258, 27 and 30 mm for *A. fumigatus* ATCC 204305, and 27 and 30 mm for *A. flavus* ATCC 204304. The respective results obtained at 48 h were 33 and 30 mm for *C. parapsilosis* 22019, 30 and 24 mm for *C. krusei* 6258, 25 and 29 mm for *A. fumigatus* ATCC 204305, and 25 and 29 mm for *A. flavus* ATCC 204304. The regression statistics are shown in Fig. 1 through 4, which demonstrate the excellent

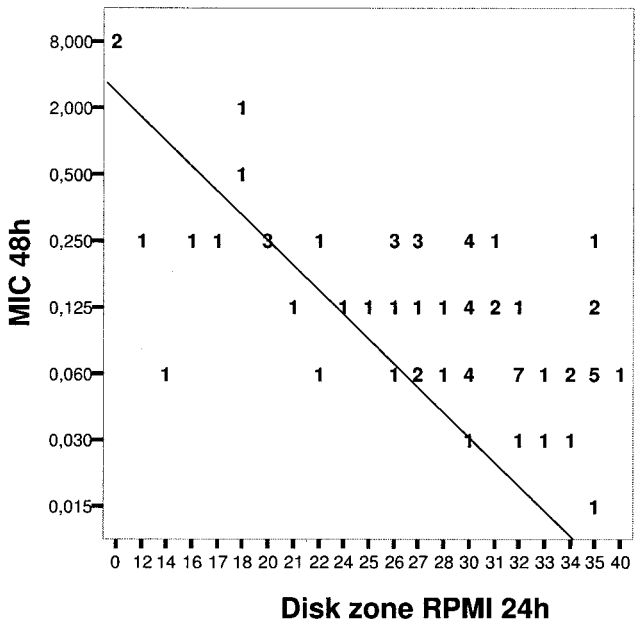

FIG. 1. Regression analysis correlating 24-h zones of inhibition (mm) determined by RPMI-G with 48-h MICs determined by the CLSI broth microdilution method (μg/ml). The regression statistics were $R = -0.690$ and $R^2 = 0.476$. Numbers within the figure are the numbers of strains that correlate with each value.

correlation between the 24- and 48-h IZ-0 results relative to the 48-h MIC-0 results. When all strains were considered together, the results for IZ-0 that were obtained with the two media were not significantly different. The results for IZ-0 that were obtained by disk diffusion showed an inverse correlation

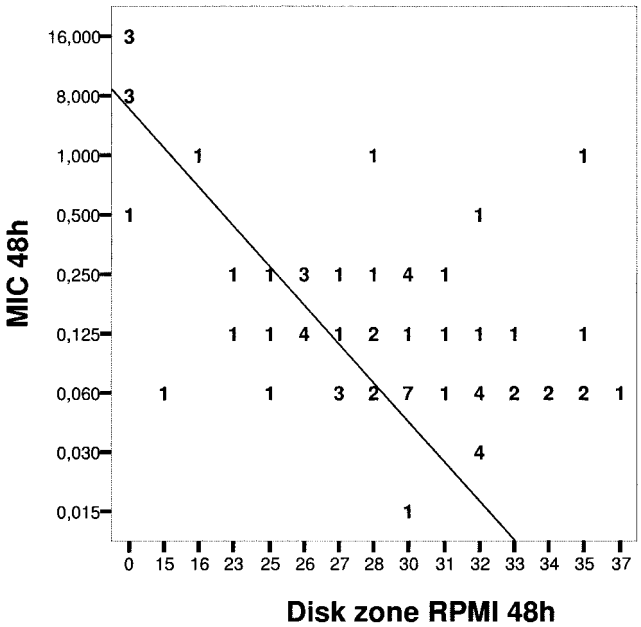

FIG. 2. Regression analysis correlating 48-h zones of inhibition (mm) determined by RPMI-G with 48-h MICs determined by the CLSI broth microdilution method (μg/ml). The regression statistics were $R = -0.803$ and $R^2 = 0.645$.

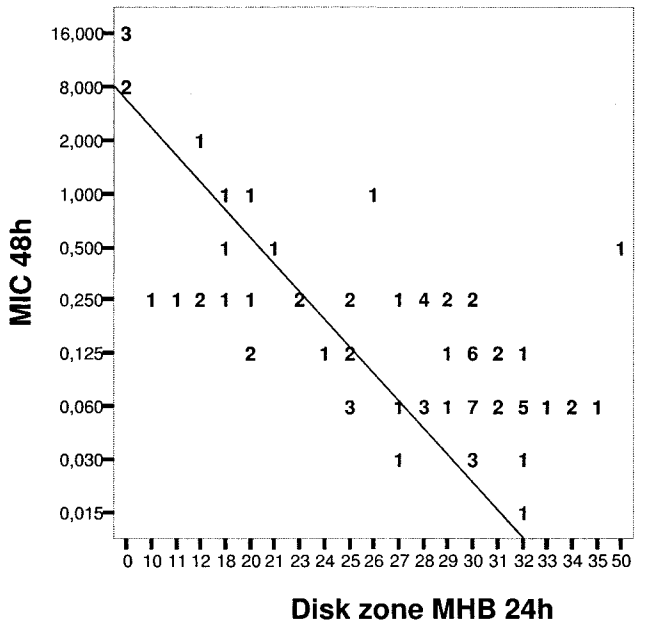

FIG. 3. Regression analysis correlating 24-h zones of inhibition (mm) determined by MHB with 48-h MICs determined by the CLSI broth microdilution method (μg/ml). The regression statistics were $R = -0.722$ and $R^2 = 0.521$.

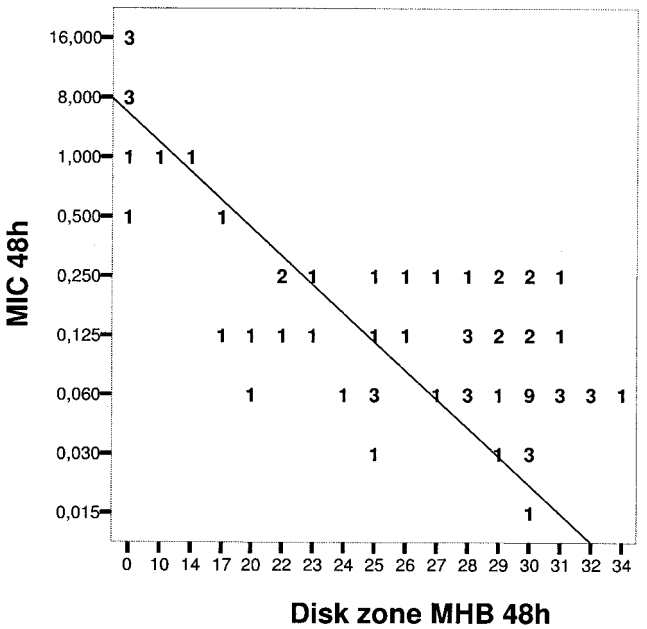

FIG. 4. Regression analysis correlating 48-h zones of inhibition (mm) determined by MHB with 48-h MICs determined by the CLSI broth microdilution method (μg/ml). The regression statistics were $R = -0.746$ and $R^2 = 0.557$.

with those that were obtained with MICs by the microdilution method. (Values for *R* [Pearson's correlation] are as follows: RPMI-G 24 [correlation between 24-h zones of inhibition {mm} determined by RPMI-G with 48-h MICs by microdilution method], −0.690; RPMI-G 48, −0.803; MHB 24, −0.722; MHB 48, −0.746.) Similarly, no differences were found between the IZ-0 results that were obtained with MHB when each species was studied separately, except for *Scedosporium* spp., which showed no growth at 24 h in RPMI-G but showed a good growth in MHB. For *Aspergillus* isolates, lower MICs correlated with the production of measurable IZs-0 around the posaconazole disks. No diameter pattern was observed to differentiate the *Aspergillus* species. For *Fusarium* spp. and *S. prolificans*, the comparison of the results that were obtained by the microdilution and disk diffusion assays showed that the high MICs obtained by the microdilution method correlated very well with the absence of IZs on disk diffusion agar plates in both media.

To our knowledge, this is the first report that uses the disk diffusion assay for susceptibility testing of posaconazole against filamentous fungi isolates and which compares the results with the microdilution method. For this reason, our results are difficult to compare with those of other authors who also tested filamentous fungi, because they used other drugs. Although the CLSI recommends using Mueller-Hinton medium for the agar test to yeasts, there is no agreement on the medium to use for filamentous fungi and as a consequence, in this work, we have evaluated two media. The disk diffusion results in RPMI-G and MHB showed a good correlation with those obtained by the CLSI method. Similar results were obtained for voriconazole against *Aspergillus* spp. by Serrano et al. (13) with MHB. The disk diffusion method is less labor intensive than the broth microdilution method and it is an attractive alternative for determining susceptibility to posaconazole because of its simplicity and low cost. Besides, it allows us to obtain the results earlier because it is possible to do the reading at 24 h of incubation. However the inability of RPMI-G to determine the susceptibility test result at 24 h for *Scedosporium* spp. appears to be a limitation of this medium. Additional studies with a higher number of strains are needed.

In this study, we obtained a good correlation between both methods. However, the utility of the disk diffusion method will depend on the MIC breakpoints that are selected and their corresponding diameter zone ranges. Further studies would be necessary to determine the interpretive criteria for both methods and to establish the correlation with in vivo response.

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